Microbiomes associated with bovine periodontitis and oral health

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Abstract

Periodontitis is an infectious polymicrobial, immuno-inflammatory disease of multifactorial aetiology that has an impact on the health, production and welfare of ruminants. The objective of the present study was to determine the microbial profiles present in the gingival sulcus of cattle considered periodontally healthy and in the periodontal pocket of animals with periodontitis lesions using high-throughput bacterial 16S rRNA gene sequencing. Subgingival biofilm samples were collected from 40 cattle with periodontitis and 38 periodontally healthy animals. In total, 1923 OTUs were identified and classified into 395 genera or higher taxa. Microbial profiles in health differed significantly from periodontitis in their composition ($p < 0.0001$, F = 5.30; PERMANOVA) but no statistically significant differences were observed in the diversity of healthy and periodontitis microbiomes. The most prevalent taxa in health were Pseudomonas, Burkholderia and Actinobacteria, whereas in disease these were Prevotella, Fusobacterium and Porphyromonas. The most discriminative taxa in health were Gastranaerophilales, Planifilum and Burkholderia, and in disease these were Elusimicrobia, Synergistes and Propionivibrio. In conclusion, statistically significant difference exists between the microbiome in bovine oral health and periodontitis, with populations showing 72.6% dissimilarity. The diversity of the bacteria found in health and periodontitis were similar and bacteria recognised as periodontal pathogens showed increased abundance in disease. In this context, the main components of bacterial homeostasis in the biofilm of healthy sites and of dysbiosis in periodontal lesions provide unprecedented indicators for the evolution of knowledge about bovine periodontitis.

Keywords: periodontal disease; cattle; high-throughput sequencing; microbiome; bacteria

1. Introduction

Periodontitis is a polymicrobial infectious disease initiated by a synergistic and dysbiotic microbial community (Hajishengallis and Lamont, 2012) that affects the health, production and welfare of ruminants. Usually neglected in animal production, it is a purulent, chronic and progressive infectious process that causes cumulative changes that occur throughout the lives of animals that is characterised by periodontal pocket formation, gingival recession, mobility, loss of clinical insertion and premature tooth loss (Page and Schroeder, 1976; Döbereiner et al., 2000; Borsanelli et al., 2016a).

The natural occurrence of periodontal lesions in sheep and cattle has been recorded in several countries and epidemiological contexts (Aitchison and Spence, 1984; Döbereiner et al., 2000; Ingham, 2001; Fadden et al., 2015; Borsanelli et al., 2016a).

Some species of oral bacteria, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, have been considered to be important in the development of periodontal disease in humans and other animal species. In cattle, the participation of some potential periodontopathogens in lesions of the disease has also been recognized, including *Fusobacterium nucleatum, Trueperella pyogenes* and some species of the *Porphyromonas, Prevotella* and *Treponema* genera (Blobel et al., 1987; Dutra et al., 2000; Borsanelli et al., 2015a, 2015b).

An important step for understanding the participation of putative bacterial pathogens in periodontitis is to determine the bacterial composition in the healthy gingival sulcus and in the periodontal pocket. It has been estimated that approximately 50% of the human oral microbiota is uncultivable (Socransky et al., 1963), and an analogous situation is likely in the bovine oral cavity.

At present, it is possible to determine almost all the community of commensal and potentially pathogenic bacteria that inhabit the bovine oral cavity, both in health and in periodontitis, using culture-independent methods. Bacterial 16S rRNA gene sequencing allows detection of not only cultivable species but also uncultivable bacteria and novel species that may be important in disease pathogenesis. This method has already been used to determine the oral bacterial community of horses,

sheep and dogs with and without periodontal lesions (Riggio et al., 2011, 2013; Kennedy et al., 2016) and to determine the oral microbiome of periodontally healthy dogs and cats (Dewhirst et al., 2012; Holcombe et al., 2014; Sturgeon et al., 2014).

The objective of the present study was to determine the microbial profiles present in the gingival sulcus of cattle considered periodontally healthy and in the periodontal pockets of animals with periodontitis lesions using high-throughput bacterial 16S rRNA gene sequencing.

2. Materials and methods

2.1. Collection of dental plaque

Two-hundred dental arches of bovines were examined at a local slaughterhouse in Scotland during the period September to November 2015 and dental plaque samples were collected. Since periodontitis includes inflammatory alterations of the gingival tissue and a progressive loss of periodontal attachment and alveolar bone, the criteria for the diagnosis of the disease was the presence of gingival retraction (i.e. the tooth root was visible at the gingival margin), the existence of a periodontal pocket (the distance from the gingival margin to the bottom of the periodontal pocket as measured with a graduated universal periodontal probe) greater than 5 mm in depth and suppuration (presence of pus inside the periodontal pocket; usually observed when curetting the bottom of the pocket). Since samples were collected post-mortem it was not possible to evaluate bleeding on probing. The periodontally healthy group had no evidence of gingival retraction, no periodontal pockets, no suppuration and no evidence of any other oral disease. The probe was inserted to the base of the periodontal pocket, applying a light force and moved gently around the tooth surface and pocket depth measurement obtained. Samples were collected within 30 minutes of death.

Subgingival plaque was collected from the periodontal pocket of 40 cattle with periodontitis and from the gingival margin around premolar 2-premolar 3 of 38 periodontally healthy cattle with

104 the aid of a sterile curette. All samples were placed in 250 µL of RNAlater (Sigma-Aldrich,
105 Gillingham, UK) and stored at -20°C until required.
106
107 *2.2. DNA preparation*
108 Subgingival plaque samples were mixed by vortexing for 30 s. To 150 µL of each sample was
109 added 200 µL phenol saturated with Tris-HCl (pH 8.0), 200 µL lysis buffer and 250 µL glass beads
110 (0.1 mm) suspended in TE buffer. Bead beating was conducted in a BioSpec Mini-Beadbeater for 2
111 min at 2100 oscillations/min. DNA was then purified using the AGOWA mag Mini DNA Isolation
112 Kit (AGOWA, Berlin, Germany).
113
114 *2.3. High-throughput sequencing*
115 Bacterial 16S rRNA genes were amplified using primers 341F (CCTACGGGNGGCWGCAG)
116 and 806R (GGACTACHVGGGTWTCTAAT) that target the V3-V4 region. Amplicon libraries were
117 purified, analysed and paired-end (2 x 301 bp) sequenced using the Illumina MiSeq as described
118 previously (Kennedy et al., 2016).
119
120 *2.4. Bioinformatics analysis*
121 USEARCH version 8.0.1623 (Edgar and Flyvbjerg, 2015) was used to merge, process and
122 cluster sequencing reads. Following merging, quality filtering (maximum expected error rate 0.002
123 and no ambiguous bases allowed) was conducted and sequences clustered into operational taxonomic
124 units (OTUs) using the settings: uparse_maxdball 1500, only *de novo* chimera checking,
125 usearch_global with -maxaccepts 8 -maxrejects 64 -maxhits 1. The most abundant sequence of each
126 OTU was selected using QIIME version 1.8.0 (Caporaso et al., 2010) and a taxonomy was then
127 assigned with the RDP classifier (Cole et al., 2009) with a minimum confidence of 0.8 and the 97%
128 representative sequence set based on the SILVA rRNA database, release 119 for QIIME (Quast et
129 al., 2013).

130 *2.5. Statistical analysis*

131 Normalisation of sequencing depth was achieved by random sub-sampling of the dataset to
132 50%. Diversity analysis (Shannon Diversity Index, Chao-1 estimate of total species richness), data
133 ordination by principal component analysis (PCA) and assessment of differences between microbial
134 profiles of the two groups by one-way PERMANOVA were performed using PAleontological
135 STatistics (PAST; v3.02) software (Hammer et al., 2001). PERMANOVA was used with Bray-Curtis
136 similarity distance. For PCA, the OTU dataset was additionally normalised by $\log_2$-transformation.
137 Diversity output was compared using the Mann-Whitney U test in SPSS (version 21.0). Linear
138 discriminant analysis effect size (LEfSe) was used to determine which OTUs and taxa contribute to
139 differences between the groups (Segata et al., 2011).

140

141 **3. Results**

142

143 *3.1. Sequencing output*

144 Sequencing generated 1,296,437 read pairs and after merging and quality filtering 86.5% of
145 these (i.e. 1,122,045) remained. Following clustering (including chimera checking) 88.5% (992,913)
146 of these 1,122,045 sequences were mapped to OTUs and were thus present in the OTU table used for
147 downstream analysis. After random subsampling at 50%, 1923 OTUs were identified and classified
148 into 395 genera or higher taxa. The most prevalent genera or higher taxa are shown in Figure 1.

149

150 *3.2. Microbial profile analysis*

151 Differences between the bovine oral microbiomes of oral health and periodontitis were evident
152 as determined by principal component analysis (Figure 2). Generally, the healthy and periodontitis
153 samples tended to cluster separately and the healthy samples demonstrated lower intra-sample
154 variability relative to the periodontitis samples. A statistically significant difference between the
155 microbial profiles of health and disease was observed ($p < 0.001$, $F = 5.30$, PERMANOVA). Bray-

Curtis analysis demonstrated 72.6% dissimilarity between the two groups. No statistically significant differences were observed in species richness or diversity of healthy and periodontitis microbiomes (Figure 3).

On average, healthy samples contained 238 OTUs (SD 158, range 66-698), while the periodontitis samples contained 245 OTUs (SD 114, range 79-577).

3.3. Differences in composition between healthy and periodontitis samples

From 395 genera or higher taxa, 45 taxa were statistically significantly different between the two groups ($p<0.05$); of these, 25 taxa had a linear discriminant analysis (LDA) score above 2 and the majority (17 of 25 taxa) were associated with disease (Figure 4). Taxa are ranked by the effect size in LEfSe.

The most discriminative taxa in the samples of healthy animals were Gastranaerophilales, Planifilum, Burkholderia and Arcobacter; in animals with periodontitis, the most discriminative taxa were Elusimicrobia, Synergistes, Propionivibrio and Fusobacteria (Figure 4).

4. Discussion

The present study is the first to use high-throughput 16S rRNA gene sequencing to compare bacterial populations present in bovine oral health and periodontitis. It was shown that a statistically significant difference exists between the microbiome in bovine oral health and periodontitis, with populations showing 72.6% dissimilarity. This represents a considerable advance in knowledge over what was previously documented for the oral microbial communities of cattle.

The human and animal oral cavity houses a complex and diverse microbial community that plays a critical role in health and disease. To date, approximately 700 species have been described in the human oral cavity, of which approximately 32% have not yet been cultivated (Chen et al., 2010). Recent advances in gene sequencing and bioinformatics technology have enabled the taxonomic

identification of previously unknown microorganisms and made it possible to more accurately describe the richness and diversity of a specific microbiome, essentially superseding Sanger 16S rRNA gene sequencing for bacterial community analysis.

Bacterial 16S rRNA gene sequencing has been used to elucidate the composition of the oral microbiome of some other animal species. Kennedy et al. (2016) identified 1308 operative taxonomic units in the oral microbiome of horses. The genera Gemella and Actinobacillus were the most abundant in samples of periodontally healthy animals, whereas in the group of animals with periodontitis the genera Prevotella and Veillonella prevailed.

In periodontally healthy dogs, Dewhirst et al. (2012) identified 353 taxa, which were placed in 14 bacterial phyla, 23 classes, 37 orders, 66 families, and 148 genera. Eighty percent of identified taxa were unnamed. Holcombe et al. (2014) evaluated the colonisation of the supragingival surface of canine teeth and identified a total of 134 species-level operative taxonomic units that were distributed among the phyla Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and Fusobacteria.

In cats, Sturgeon et al. (2014) identified 10177 OTUs in the oral microbiome of healthy animals, representing 18 phyla, of which the most prevalent were Proteobacteria (75.2%), Bacteroidetes (9.3%), Firmicutes (6.7%), Spirochaetes (1.8%), Fusobacteria (1.3%) and Actinobacteria (0.6%). The most prevalent genera were Moraxella (10.9%), Thermomonas (6.9%), Neisseria (4.9%) and Pasteurella (4.3%).

In the present study, the taxa Gastranaerophilales, Planifilum, Burkholderia and Arcobacter were the most prevalent in healthy animals, while Elusimicrobia, Synergistes and Propionivibrio were most frequently observed in the oral microbiota of cattle with periodontitis. However, little is known regarding these microorganisms. Fusobacteria, Wolinella, Porphyromonas, Prevotella and Treponema were also found at high prevalence in bovine periodontitis lesions.

The Fusobacteria phylum, which contains bacteria of the genus Fusobacterium, has been recognised as part of the subgingival microbiota for more than 100 years. In cattle, *Fusobacterium*

208 *nucleatum* was detected in culture of periodontitis lesions (Blobel et al., 1987; Botteon et al., 1993).
209 *Fusobacterium naviforme, Fusobacterium necrophorum* and *F. nucleatum* have been identified in
210 sheep with 'broken mouth' periodontitis (McCourtie et al., 1989) and *F. necrophorum* has been
211 identified in goats with periodontitis (Suzuki et al., 2006).

212 The Fusobacterium genus is one of the main constituents of the normal oral microbiota of cats
213 (Love et al., 1990) and several species of the genus, such as *Fusobacterium alocis* (Hardham et al.,
214 2005), *Fusobacterium canifelinum* (Conrads et al., 2004; Dahlén et al., 2012) and *F. nucleatum*
215 (Nishiyama et al., 2007), have been detected in dogs with and without periodontitis.

216 Black-pigmented bacteria of the genera Porphyromonas and Prevotella are recognised
217 pathogens in human and animal periodontitis. Different species of both genera have been identified
218 in dogs (Hardham et al., 2005; Nishiyama et al., 2007; Riggio et al., 2011), cats (Booij-Vrieling et
219 al., 2010) and sheep with periodontitis (McCourtie et al., 1989, Duncan et al., 2003; Riggio et al.,
220 2013; Borsanelli et al., 2017).

221 In cattle, these two genera appear to play an important role in the lesions of animals with
222 periodontitis (Blobel et al., 1987; Botteon et al., 1993; Dutra et al., 1986, 2000). When evaluating the
223 presence of Prevotella and Porphyromonas species in the bovine microbiota with and without
224 periodontitis, Borsanelli et al. (2015b) found that the occurrence of *Porphyromonas asaccharolytica*,
225 *Porphyromonas endodontalis*, *Prevotella buccae*, *Prevotella intermedia*, *Prevotella melaninogenica*
226 and *Prevotella oralis* was associated with bovine periodontitis.

227 There are a variety of quantitative and qualitative studies that evaluated Treponema species
228 involved in human periodontitis or healthy sites (Sato and Kuramitsu, 2000; Asai et al., 2002), as
229 well as in dogs with periodontitis (Riviere et al., 1996; Nordhoff et al., 2008). Several species of the
230 genus Treponema were identified in periodontal lesions of sheep (Borsanelli et al., 2016b), and in
231 cattle *Treponema amylovorum*, *Treponema maltophilum* and *Treponema denticola* were detected in
232 the microbiota of animals with periodontitis (Borsanelli et al., 2015a). This genus was also found at
233 high levels in horses with periodontitis (Kennedy et al., 2016).

No previous study has characterised the bovine oral microbiome in as much detail as presented in the current study. Samples from bovine oral health and periodontitis had different microbial profiles, but the diversity of the bacteria found in health and periodontitis were similar; bacteria commonly recognised as periodontal pathogens showed an increased abundance in disease. In this context, the main components of bacterial homeostasis in the biofilm of healthy sites and of dysbiosis in periodontal lesions provide unprecedented indicators for the evolution of knowledge about bovine periodontitis.

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Conflicts of interest statement

The authors have no conflicts of interest.

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Figure 1. Prevalence of genera or higher taxa in oral health and bovine periodontitis.

Distribution of the most prevalent genera or higher taxa in healthy and periodontitis samples from cattle. The average number of OTUs per sample representing each taxon are shown for health (green) and periodontitis (red).

Figure 2. Two-dimensional ordination of bovine microbial profiles in oral health and periodontitis by principal component analysis (PCA).

Identified OTUs were randomly subsampled to 50% and $\log_2$-transformed prior to the PCA.

Figure 3. Diversity analysis in bovine microbial profiles at health and periodontitis.

A. Observed species richness or number of OTUs per sample; B. Estimated species richness or Chao-1; C. Shannon diversity index.

Figure 4. Visualisation of most significant taxa (genus or higher level) that differentiate between health and periodontitis in bovine microbiomes.

Forty-five taxa were statistically significantly different between the two groups. Only taxa with an LDA score of two or above are shown. Taxa are ranked by the effect size in LEfSe.

- First study to investigate the microbiomes of bovine oral health and periodontitis
- Microbiomes of health and periodontitis show a dissimilarity of 72.6%
- Pseudomonas, Burkholderia and Actinobacteria are associated with oral health
- Prevotella, Fusobacterium and Porphyromonas are associated with periodontitis

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Abstract

Periodontitis is an infectious polymicrobial, immuno-inflammatory disease of multifactorial aetiology that has an impact on the health, production and welfare of ruminants. The objective of the present study was to determine the microbial profiles present in the gingival sulcus of cattle considered periodontally healthy and in the periodontal pocket of animals with periodontitis lesions using high-throughput bacterial 16S rRNA gene sequencing. Subgingival biofilm samples were collected from 40 cattle with periodontitis and 38 periodontally healthy animals. In total, 1923 OTUs were identified and classified into 395 genera or higher taxa. Microbial profiles in health differed significantly from periodontitis in their composition ($p < 0.0001$, F = 5.30; PERMANOVA) but no statistically significant differences were observed in the diversity of healthy and periodontitis microbiomes. The most prevalent taxa in health were Pseudomonas, Burkholderia and Actinobacteria, whereas in disease these were Prevotella, Fusobacterium and Porphyromonas. The most discriminative taxa in health were Gastranaerophilales, Planifilum and Burkholderia, and in disease these were Elusimicrobia, Synergistes and Propionivibrio. In conclusion, statistically significant difference exists between the microbiome in bovine oral health and periodontitis, with populations showing 72.6% dissimilarity. The diversity of the bacteria found in health and periodontitis were similar and bacteria recognised as periodontal pathogens showed increased abundance in disease. In this context, the main components of bacterial homeostasis in the biofilm of healthy sites and of dysbiosis in periodontal lesions provide unprecedented indicators for the evolution of knowledge about bovine periodontitis.

Keywords: periodontal disease; cattle; high-throughput sequencing; microbiome; bacteria

1. Introduction

Periodontitis is a polymicrobial infectious disease initiated by a synergistic and dysbiotic microbial community (Hajishengallis and Lamont, 2012) that affects the health, production and welfare of ruminants. Usually neglected in animal production, it is a purulent, chronic and progressive infectious process that causes cumulative changes that occur throughout the lives of animals that is characterised by periodontal pocket formation, gingival recession, mobility, loss of clinical insertion and premature tooth loss (Page and Schroeder, 1976; Döbereiner et al., 2000; Borsanelli et al., 2016a).

The natural occurrence of periodontal lesions in sheep and cattle has been recorded in several countries and epidemiological contexts (Aitchison and Spence, 1984; Döbereiner et al., 2000; Ingham, 2001; Fadden et al., 2015; Borsanelli et al., 2016a).

Some species of oral bacteria, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, have been considered to be important in the development of periodontal disease in humans and other animal species. In cattle, the participation of some potential periodontopathogens in lesions of the disease has also been recognized, including *Fusobacterium nucleatum, Trueperella pyogenes* and some species of the *Porphyromonas, Prevotella* and *Treponema* genera (Blobel et al., 1987; Dutra et al., 2000; Borsanelli et al., 2015a, 2015b).

An important step for understanding the participation of putative bacterial pathogens in periodontitis is to determine the bacterial composition in the healthy gingival sulcus and in the periodontal pocket. It has been estimated that approximately 50% of the human oral microbiota is uncultivable (Socransky et al., 1963), and an analogous situation is likely in the bovine oral cavity.

At present, it is possible to determine almost all the community of commensal and potentially pathogenic bacteria that inhabit the bovine oral cavity, both in health and in periodontitis, using culture-independent methods. Bacterial 16S rRNA gene sequencing allows detection of not only cultivable species but also uncultivable bacteria and novel species that may be important in disease pathogenesis. This method has already been used to determine the oral bacterial community of horses,

sheep and dogs with and without periodontal lesions (Riggio et al., 2011, 2013; Kennedy et al., 2016) and to determine the oral microbiome of periodontally healthy dogs and cats (Dewhirst et al., 2012; Holcombe et al., 2014; Sturgeon et al., 2014).

The objective of the present study was to determine the microbial profiles present in the gingival sulcus of cattle considered periodontally healthy and in the periodontal pockets of animals with periodontitis lesions using high-throughput bacterial 16S rRNA gene sequencing.

2. Materials and methods

2.1. Collection of dental plaque

Two-hundred dental arches of bovines were examined at a local slaughterhouse in Scotland during the period September to November 2015 and dental plaque samples were collected. Since periodontitis includes inflammatory alterations of the gingival tissue and a progressive loss of periodontal attachment and alveolar bone, the criteria for the diagnosis of the disease was the presence of gingival retraction (i.e. the tooth root was visible at the gingival margin), the existence of a periodontal pocket (the distance from the gingival margin to the bottom of the periodontal pocket as measured with a graduated universal periodontal probe) greater than 5 mm in depth and suppuration (presence of pus inside the periodontal pocket; usually observed when curetting the bottom of the pocket). Since samples were collected post-mortem it was not possible to evaluate bleeding on probing. The periodontally healthy group had no evidence of gingival retraction, no periodontal pockets, no suppuration and no evidence of any other oral disease. The probe was inserted to the base of the periodontal pocket, applying a light force and moved gently around the tooth surface and pocket depth measurement obtained. Samples were collected within 30 minutes of death.

Subgingival plaque was collected from the periodontal pocket of 40 cattle with periodontitis and from the gingival margin around premolar 2-premolar 3 of 38 periodontally healthy cattle with

the aid of a sterile curette. All samples were placed in 250 µL of RNAlater (Sigma-Aldrich, Gillingham, UK) and stored at -20°C until required.

2.2. DNA preparation

Subgingival plaque samples were mixed by vortexing for 30 s. To 150 µL of each sample was added 200 µL phenol saturated with Tris-HCl (pH 8.0), 200 µL lysis buffer and 250 µL glass beads (0.1 mm) suspended in TE buffer. Bead beating was conducted in a BioSpec Mini-Beadbeater for 2 min at 2100 oscillations/min. DNA was then purified using the AGOWA mag Mini DNA Isolation Kit (AGOWA, Berlin, Germany).

2.3. High-throughput sequencing

Bacterial 16S rRNA genes were amplified using primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTWTCTAAT) that target the V3-V4 region. Amplicon libraries were purified, analysed and paired-end (2 x 301 bp) sequenced using the Illumina MiSeq as described previously (Kennedy et al., 2016).

2.4. Bioinformatics analysis

USEARCH version 8.0.1623 (Edgar and Flyvbjerg, 2015) was used to merge, process and cluster sequencing reads. Following merging, quality filtering (maximum expected error rate 0.002 and no ambiguous bases allowed) was conducted and sequences clustered into operational taxonomic units (OTUs) using the settings: uparse_maxdball 1500, only *de novo* chimera checking, usearch_global with -maxaccepts 8 -maxrejects 64 -maxhits 1. The most abundant sequence of each OTU was selected using QIIME version 1.8.0 (Caporaso et al., 2010) and a taxonomy was then assigned with the RDP classifier (Cole et al., 2009) with a minimum confidence of 0.8 and the 97% representative sequence set based on the SILVA rRNA database, release 119 for QIIME (Quast et al., 2013).

2.5. Statistical analysis

Normalisation of sequencing depth was achieved by random sub-sampling of the dataset to 50%. Diversity analysis (Shannon Diversity Index, Chao-1 estimate of total species richness), data ordination by principal component analysis (PCA) and assessment of differences between microbial profiles of the two groups by one-way PERMANOVA were performed using PAleontological STatistics (PAST; v3.02) software (Hammer et al., 2001). PERMANOVA was used with Bray-Curtis similarity distance. For PCA, the OTU dataset was additionally normalised by $\log_2$-transformation. Diversity output was compared using the Mann-Whitney U test in SPSS (version 21.0). Linear discriminant analysis effect size (LEfSe) was used to determine which OTUs and taxa contribute to differences between the groups (Segata et al., 2011).

3. Results

3.1. Sequencing output

Sequencing generated 1,296,437 read pairs and after merging and quality filtering 86.5% of these (i.e. 1,122,045) remained. Following clustering (including chimera checking) 88.5% (992,913) of these 1,122,045 sequences were mapped to OTUs and were thus present in the OTU table used for downstream analysis. After random subsampling at 50%, 1923 OTUs were identified and classified into 395 genera or higher taxa. The most prevalent genera or higher taxa are shown in Figure 1.

3.2. Microbial profile analysis

Differences between the bovine oral microbiomes of oral health and periodontitis were evident as determined by principal component analysis (Figure 2). Generally, the healthy and periodontitis samples tended to cluster separately and the healthy samples demonstrated lower intra-sample variability relative to the periodontitis samples. A statistically significant difference between the microbial profiles of health and disease was observed ($p < 0.001$, $F = 5.30$, PERMANOVA). Bray-

Curtis analysis demonstrated 72.6% dissimilarity between the two groups. No statistically significant differences were observed in species richness or diversity of healthy and periodontitis microbiomes (Figure 3).

On average, healthy samples contained 238 OTUs (SD 158, range 66-698), while the periodontitis samples contained 245 OTUs (SD 114, range 79-577).

3.3. Differences in composition between healthy and periodontitis samples

From 395 genera or higher taxa, 45 taxa were statistically significantly different between the two groups ($p<0.05$); of these, 25 taxa had a linear discriminant analysis (LDA) score above 2 and the majority (17 of 25 taxa) were associated with disease (Figure 4). Taxa are ranked by the effect size in LEfSe.

The most discriminative taxa in the samples of healthy animals were Gastranaerophilales, Planifilum, Burkholderia and Arcobacter; in animals with periodontitis, the most discriminative taxa were Elusimicrobia, Synergistes, Propionivibrio and Fusobacteria (Figure 4).

4. Discussion

The present study is the first to use high-throughput 16S rRNA gene sequencing to compare bacterial populations present in bovine oral health and periodontitis. It was shown that a statistically significant difference exists between the microbiome in bovine oral health and periodontitis, with populations showing 72.6% dissimilarity. This represents a considerable advance in knowledge over what was previously documented for the oral microbial communities of cattle.

The human and animal oral cavity houses a complex and diverse microbial community that plays a critical role in health and disease. To date, approximately 700 species have been described in the human oral cavity, of which approximately 32% have not yet been cultivated (Chen et al., 2010). Recent advances in gene sequencing and bioinformatics technology have enabled the taxonomic

identification of previously unknown microorganisms and made it possible to more accurately describe the richness and diversity of a specific microbiome, essentially superseding Sanger 16S rRNA gene sequencing for bacterial community analysis.

Bacterial 16S rRNA gene sequencing has been used to elucidate the composition of the oral microbiome of some other animal species. Kennedy et al. (2016) identified 1308 operative taxonomic units in the oral microbiome of horses. The genera Gemella and Actinobacillus were the most abundant in samples of periodontally healthy animals, whereas in the group of animals with periodontitis the genera Prevotella and Veillonella prevailed.

In periodontally healthy dogs, Dewhirst et al. (2012) identified 353 taxa, which were placed in 14 bacterial phyla, 23 classes, 37 orders, 66 families, and 148 genera. Eighty percent of identified taxa were unnamed. Holcombe et al. (2014) evaluated the colonisation of the supragingival surface of canine teeth and identified a total of 134 species-level operative taxonomic units that were distributed among the phyla Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and Fusobacteria.

In cats, Sturgeon et al. (2014) identified 10177 OTUs in the oral microbiome of healthy animals, representing 18 phyla, of which the most prevalent were Proteobacteria (75.2%), Bacteroidetes (9.3%), Firmicutes (6.7%), Spirochaetes (1.8%), Fusobacteria (1.3%) and Actinobacteria (0.6%). The most prevalent genera were Moraxella (10.9%), Thermomonas (6.9%), Neisseria (4.9%) and Pasteurella (4.3%).

In the present study, the taxa Gastranaerophilales, Planifilum, Burkholderia and Arcobacter were the most prevalent in healthy animals, while Elusimicrobia, Synergistes and Propionivibrio were most frequently observed in the oral microbiota of cattle with periodontitis. However, little is known regarding these microorganisms. Fusobacteria, Wolinella, Porphyromonas, Prevotella and Treponema were also found at high prevalence in bovine periodontitis lesions.

The Fusobacteria phylum, which contains bacteria of the genus Fusobacterium, has been recognised as part of the subgingival microbiota for more than 100 years. In cattle, *Fusobacterium*

nucleatum was detected in culture of periodontitis lesions (Blobel et al., 1987; Botteon et al., 1993). *Fusobacterium naviforme, Fusobacterium necrophorum* and *F. nucleatum* have been identified in sheep with 'broken mouth' periodontitis (McCourtie et al., 1989) and *F. necrophorum* has been identified in goats with periodontitis (Suzuki et al., 2006).

The Fusobacterium genus is one of the main constituents of the normal oral microbiota of cats (Love et al., 1990) and several species of the genus, such as *Fusobacterium alocis* (Hardham et al., 2005), *Fusobacterium canifelinum* (Conrads et al., 2004; Dahlén et al., 2012) and *F. nucleatum* (Nishiyama et al., 2007), have been detected in dogs with and without periodontitis.

Black-pigmented bacteria of the genera Porphyromonas and Prevotella are recognised pathogens in human and animal periodontitis. Different species of both genera have been identified in dogs (Hardham et al., 2005; Nishiyama et al., 2007; Riggio et al., 2011), cats (Booij-Vrieling et al., 2010) and sheep with periodontitis (McCourtie et al., 1989, Duncan et al., 2003; Riggio et al., 2013; Borsanelli et al., 2017).

In cattle, these two genera appear to play an important role in the lesions of animals with periodontitis (Blobel et al., 1987; Botteon et al., 1993; Dutra et al., 1986, 2000). When evaluating the presence of Prevotella and Porphyromonas species in the bovine microbiota with and without periodontitis, Borsanelli et al. (2015b) found that the occurrence of *Porphyromonas asaccharolytica*, *Porphyromonas endodontalis*, *Prevotella buccae*, *Prevotella intermedia*, *Prevotella melaninogenica* and *Prevotella oralis* was associated with bovine periodontitis.

There are a variety of quantitative and qualitative studies that evaluated Treponema species involved in human periodontitis or healthy sites (Sato and Kuramitsu, 2000; Asai et al., 2002), as well as in dogs with periodontitis (Riviere et al., 1996; Nordhoff et al., 2008). Several species of the genus Treponema were identified in periodontal lesions of sheep (Borsanelli et al., 2016b), and in cattle *Treponema amylovorum*, *Treponema maltophilum* and *Treponema denticola* were detected in the microbiota of animals with periodontitis (Borsanelli et al., 2015a). This genus was also found at high levels in horses with periodontitis (Kennedy et al., 2016).

No previous study has characterised the bovine oral microbiome in as much detail as presented in the current study. Samples from bovine oral health and periodontitis had different microbial profiles, but the diversity of the bacteria found in health and periodontitis were similar; bacteria commonly recognised as periodontal pathogens showed an increased abundance in disease. In this context, the main components of bacterial homeostasis in the biofilm of healthy sites and of dysbiosis in periodontal lesions provide unprecedented indicators for the evolution of knowledge about bovine periodontitis.

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Conflicts of interest statement

The authors have no conflicts of interest.

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Figure 1. Prevalence of genera or higher taxa in oral health and bovine periodontitis.

Distribution of the most prevalent genera or higher taxa in healthy and periodontitis samples from cattle. The average number of OTUs per sample representing each taxon are shown for health (green) and periodontitis (red).

Figure 2. Two-dimensional ordination of bovine microbial profiles in oral health and periodontitis by principal component analysis (PCA).

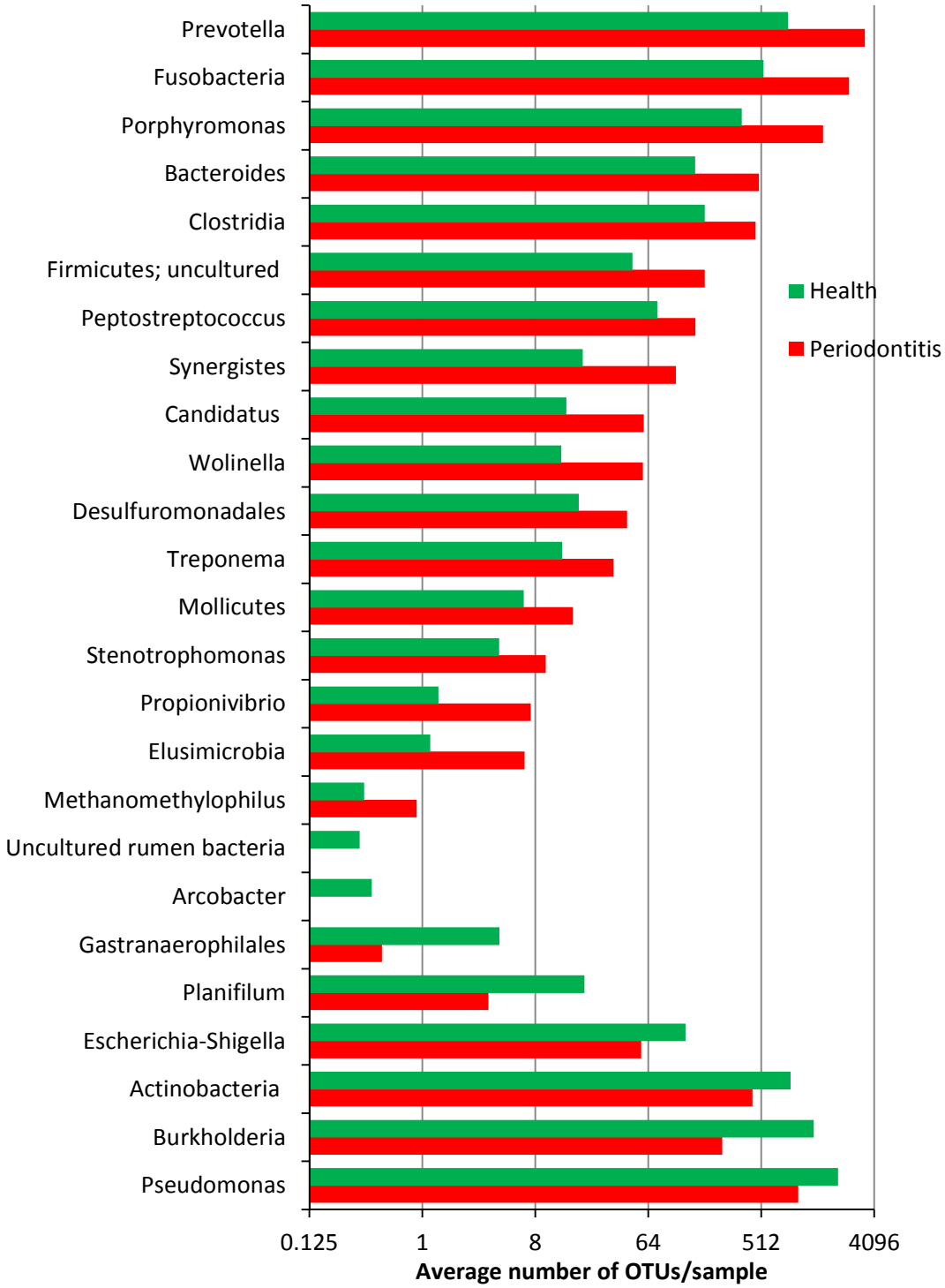
Identified OTUs were randomly subsampled to 50% and $\log_2$-transformed prior to the PCA.

Figure 3. Diversity analysis in bovine microbial profiles at health and periodontitis.

A. Observed species richness or number of OTUs per sample; B. Estimated species richness or Chao-1; C. Shannon diversity index.

Figure 4. Visualisation of most significant taxa (genus or higher level) that differentiate between health and periodontitis in bovine microbiomes.

Forty-five taxa were statistically significantly different between the two groups. Only taxa with an LDA score of two or above are shown. Taxa are ranked by the effect size in LEfSe.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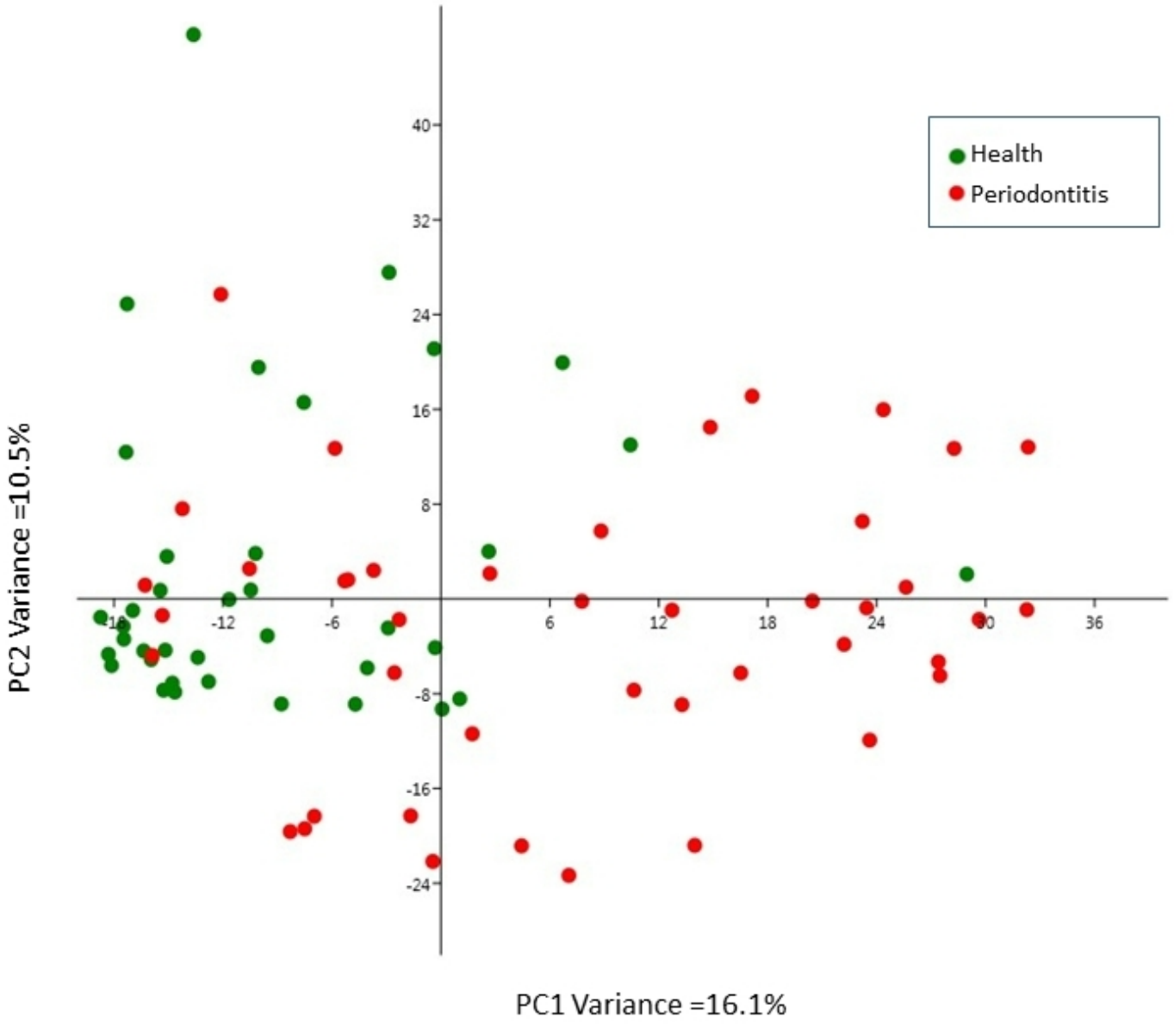
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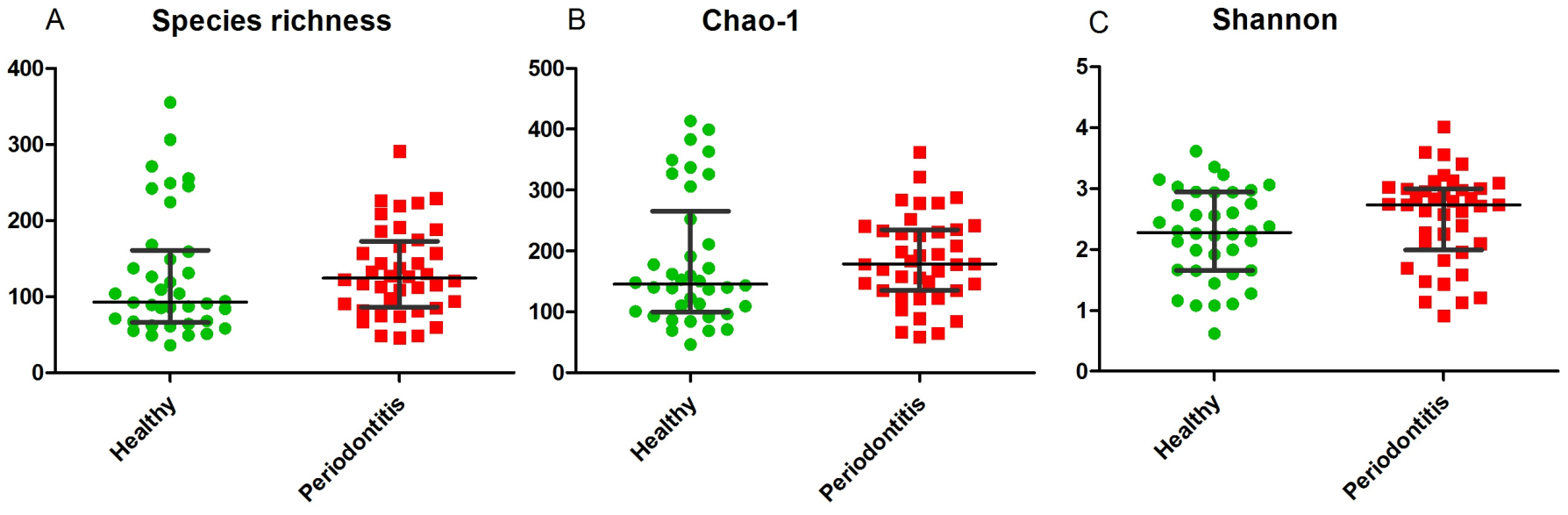
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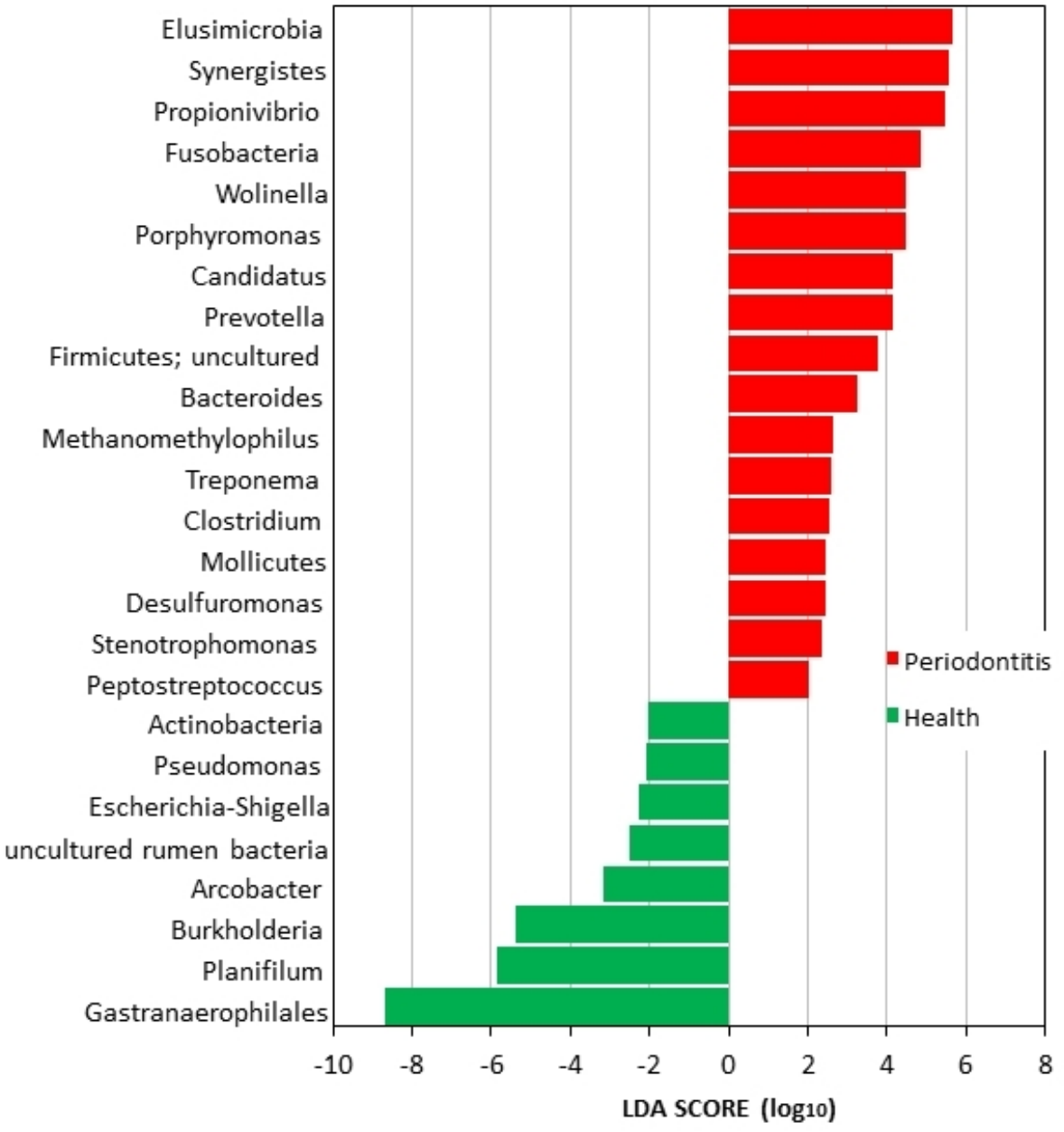
Healthy
Periodontitis


Elusimicrobia
Synergistes
Propionivibrio
Fusobacteria
Wolinella
Porphyromonas
Candidatus
Prevotella
Firmicutes; uncultured
Bacteroides
Methanomethylophilus
Treponema
Clostridium
Mollicutes
Desulfuromonas
Stenotrophomonas
Peptostreptococcus
Actinobacteria
Pseudomonas
Escherichia-Shigella
uncultured rumen bacteria
Arcobacter
Burkholderia
Planifilum
Gastranaerophilales
Periodontitis
Health
-10
-8
-6
-4
-2
0
2
4
6
8
LDA SCORE (log10)